Apigenin in Combination with Akt Inhibition Significantly Enhances Thyrotropin-stimulated Radioiodide Accumulation in Thyroid Cells

Aparna Lakshmanan[1, 3], Andrea I. Doseff [1, 2] Matthew D. Ringel[2], Motoyasu Saji[2], Bernard Rousset[4], Xiaoli Zhang[5] and Sissy M. Jhiang[1, 3]

From the [1]Molecular, Cellular and Developmental Biology Graduate Program, [2]Department of Internal Medicine and [3]Department of Physiology and Cell Biology, [5]Center for Biostatistics, The Ohio State University, Columbus, OH 43210, USA

[4]Institut National de la Santé et de la Recherche Médicale, Inserm U1052, Centre de Recherche en Cancérologie de Lyon, F-69000 Lyon, France; Université de lyon, F-69000 Lyon, France

AL: lakshmanan.10@osu.edu (B.Tech), AID: andrea.doseff@osumc.edu (PhD), MDR: matthew.ringel@osumc.edu (MD), MS: moto.saji@osumc.edu (MD, PhD), BR: bernard.rousset@univ-lyon1.fr (PhD), XZ: xiaoli.zhang@osumc.edu (PhD), SMJ: jhiang.1@osu.edu (PhD)

Running Title: Apigenin/Akt inhibition enhance thyroidal iodide uptake

Keywords: NIS, TSH, Iodine uptake and Iodine metabolism, Thyroid Cancer- Basic, Molecular Biology

Abstract:

Background: Selectively increased radioiodine accumulation in thyroid cells by thyrotropin (TSH) allows targeted treatment of thyroid cancer. However, the extent of TSH-stimulated radioiodine accumulation in some thyroid tumors is not sufficient to confer therapeutic efficacy. Hence, it is of clinical importance to identify novel strategies to selectively further enhance TSH-stimulated thyroidal radioiodine accumulation.

Methods: PCCl3 rat thyroid cells, PCCl3 cells overexpressing $BRAF^{V600E}$, or primary cultured tumor cells from a thyroid cancer mouse model, under TSH stimulation were treated with various reagents for 24 hrs. Cells were then subjected to radioactive iodide uptake, kinetics, efflux assays, and protein extraction followed by western blotting against selected antibodies.

Results: We previously reported that Akt inhibition increased radioiodine accumulation in thyroid cells under chronic TSH stimulation. Here, we identified Apigenin, a plant-derived flavonoid, as a reagent to further enhance iodide influx rate increased by Akt inhibition in thyroid cells under acute TSH stimulation. Akt inhibition is permissive for Apigenin's action, as Apigenin alone had little effect. This action of Apigenin requires p38 MAPK activity, but not PKC-δ. The increase in radioiodide accumulation by Apigenin with Akt inhibition was also observed in thyroid cells expressing $BRAF^{V600E}$ and in primary cultured thyroid tumor cells from $TR\beta^{PV/PV}$ mice.

Conclusion: Taken together, Apigenin may serve as a dietary supplement in combination with Akt inhibitors to enhance therapeutic efficacy of radioiodine for thyroid cancer.

Introduction:

The Na^+/I^- Symporter (NIS) is a glycoprotein expressed on the basolateral membrane of thyroid follicular cells that facilitates active uptake of iodide from circulating blood. The iodide is further retained in the thyroid follicle by organification, where it is incorporated into the tyrosine amino acid residues of thyroglobulin, the precursor of thyroxine (T4) and triiodothyronine (T3) thyroid hormones. Thyroidal radioiodine accumulation serves as the basis for targeted ablation of post-thyroidectomy remnants. Since radioiodine accumulation in most thyroid tumors can be further enhanced by elevation of serum thyrotropin (TSH) levels, many patients with recurrent and metastatic thyroid cancers can benefit from radioiodine therapy upon administration of recombinant human TSH or T4 withdrawal (1, 2). However, in a substantial number of patients, the extent of TSH-stimulated radioiodine accumulation is not sufficient to confer therapeutic efficacy. Thus, it is of clinical importance to identify novel strategies to selectively further enhance TSH-stimulated thyroidal radioiodine accumulation.

Pharmacological inhibitors targeting signaling pathways activated in thyroid cancers, such as, MEK/ERK (3), Hsp90 (4) and PI3K/Akt (5) have been shown to increase radioactive iodide uptake (RAIU) in PCCl3 rat thyroid cells. To date, the effect of MEK and BRAF inhibition (6, 7) and 17-AAG (4) on increasing RAI accumulation in cultured thyroid cells have been validated in mouse models of thyroid cancer (7, 8) and promising results were recently reported in a clinical trial for patients treated with a MEK inhibitor as pretreatment for I-131 therapy (9).

We examined the effects of various inhibitors on RAIU in PCCl3 cells, which have undergone TSH withdrawal for 5 days followed by acute TSH stimulation for 24 hrs prior to

treatment with inhibitors. In this experimental setting, we show that Akt inhibitor (Akti1/2) had the greatest extent of increase in RAIU and Apigenin further increased thyroidal RAIU in combination with Akti1/2. The action of Apigenin to further increase Akti1/2-induced RAIU in thyroid cells is dependent on p38 MAPK activity. Taken together, Apigenin has the potential to serve as a dietary supplement along with Akt inhibitors to increase the efficacy of radioiodine therapy for patients with advanced thyroid cancer.

Methods:

Cell culture, reagents and TRβ$^{PV/PV}$ mouse model. PCCl3 rat thyroid cells were maintained in 6H media with 5% bovine serum as described by Liu et al. (5), unless specified otherwise. Experiments were performed under acute TSH stimulation, where cells were withdrawn from TSH for 5 days (5H media) and then TSH was added back for 24 hrs prior to treatment with various reagents for additional 24 hrs, unless specified otherwise. PCCl3/TetOn-BRAF^{V600E}, a generous gift from Dr. James Fagin, Memorial Sloan Kettering Cancer Center, New York (10) and PCCl3/TetOn-PTC1 cells (4) were genetically modified from PCCl3 cells to allow doxycycline-inducible expression of BRAF^{V600E} or PTC1 oncogenes respectively. Experiments were performed under acute TSH stimulation with or without 2 μg/ml of doxycycline for 48 hrs, followed by treatment with reagents for additional 24 hrs. Primary cultured cells from mouse thyroid tumors were isolated using a tumor dissociation kit (Miltenyi Biotec Inc., Bergisch Gladbach, Germany), according to the manufacturer's protocol and were cultured in 6H media. Reagents used in this study are listed as follows: Akti1/2 also known as Akt inhibitor VIII, 17-AAG and SB203580 (EMD Millipore, Billerica, MA), LY294002 (Cayman Chemical Company, Ann Arbor, MI), PD98059 (Cell Signaling Technology Inc. Beverly, MA), Apigenin and DMSO

(Sigma-Aldrich®, St. Louis, MO), BIRB-796 (Selleck Chemicals, Houston, TX) and Silencer® select scrambled and PKC-δ siRNAs (Ambion, Austin, TX). Control vector and shAkt1/2 plasmids were generous gifts from Dr. Mingzhao Xing at The Johns Hopkins University School of Medicine. $TR\beta^{PV/PV}$ genetically engineered mice were obtained from Dr. Sheue-yann Cheng, National Cancer Institute, Bethesda (11).

RT^2 profiler PCR array and Ingenuity Pathway Analysis (IPA). **A** Rat Epithelial to Mesenchymal transition (EMT) RT^2 Profiler PCR array that profiles the expression of 84 key genes was purchased from SABiosciences, Valencia, CA. Total RNA isolated from PCCl3 cells treated with DMSO, Akti1/2, and TGF-β was reversed transcribed to cDNA and real-time PCR was performed per manufacturer's instructions. Genes with expression levels of Ct value less than 30 in either the experimental or the control group along with their fold changes were submitted for IPA analysis to predict upstream transcription factors and their activation status. Additional downstream targets of these transcription factors were identified by IPA analysis. Among targets identified, we searched for ones that are oppositely modulated by Akti1/2 and TGF-β.

Transfections. Plasmid transfections were conducted using FuGENE®6 (Promega, Madison, WI) for 24 hrs followed by replacement with fresh media for 24 hrs. Stably transfected cells were selected under media containing 800 μg/ml of G418 (Life Technologies, Carlsbad, CA) for 2 weeks with media replacement every other day. Mixed stable clones were then maintained in 400 μg/ml of G418. Transfections with siRNAs were facilitated by incubating with

Lipofectamine® RNAiMAX (Life Technologies) for 24 hrs followed by replacement with fresh media with or without drug treatments for 24 hrs.

RAIU, Kinetics and Efflux Assays. These assays were performed as previously described by Vadysirisack et al. (3) with I-125 in NaI (80mCi/mmol). Note that RAIU in all figures, except efflux assay, represents NIS-mediated RAIU acquired by subtracting background RAIU value from parallel experiments in the presence of 100 μM perchlorate. The magnitude of RAIU in the presence of perchlorate varies between 10^2-10^3 cpm among different trials. However, the background values did not vary much among various experimental groups within the same trial. Accordingly, the background measurement for each experimental group within each trial was not always replicated. The background value (or the average of replicates) was subtracted from the corresponding experimental group before statistical analysis.

Western blot analysis. Cells were lysed and subjected to gel electrophoresis and Western blot analysis as previously described (5). In this study, 4-20% gradient Tris-Glycine or Tris HEPES SDS-PAGE gels (NuSep Inc. or Bio-Rad Laboratories, Inc) were used, and the NIS protein was detected with the PA716 rNIS polyclonal antibody (provided by Dr. B. Rousset) at a dilution of 1:1500. Phospho-Akt, p38 MAPK, MAPKAPK2 and PKC-δ were detected using 1:1000 dilution of antibodies from Cell Signaling Technology Inc., (Cat. 9271, 9212, 3042) and Santa Cruz Biotechnology Inc., Dallas, TX (Cat. sc-213) respectively. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibodies were used accordingly. Equivalent protein loading among samples was monitored by probing for β-actin (Abcam plc, Cambridge, MA; Cat.

8226) or GAPDH (Cell signaling technology Inc., Cat. 2118). Densitometry analysis was performed using ImageJ software.

Cell surface biotinylation. Cell surface biotinylation was performed as previously described (3). Equivalent loading of cell surface proteins was monitored by probing for Na^+/K^+ ATPase using mouse anti-Na^+/K^+ ATPase monoclonal antibody (Santa Cruz Biotechnology Inc., Cat. sc-21712) and enrichment of cell surface proteins was confirmed by absence of β-actin in the cell surface eluate. In this study, phosphatase inhibitors were not added to cell lysis buffer.

Statistical analysis. All experiments had at least two independent trials with three replicates for each experimental group within each trial. For RAIU assay, all the data values were log10 transformed to reduce variance and skewness and then linear mixed effects models were used to take account of the correlations among observations from the same trial. From the model, all the pre-specified comparisons for each experiment were obtained and adjusted for multiple comparisons using sequentially rejective Bonferroni test (12, 13) to control type I error at 0.05. SAS 9.2 software was used for analysis (SAS Institute Inc., NC). If no significance was found, we just interpreted as no significant difference between the compared conditions instead of using the included comparisons to exclude the outcome for another comparison. GraphPad Prism version 6.0 was used to perform statistical analyses for efflux assay and to determine Km and Vmax values for kinetics assay.

Results

Akti1/2 increases TSH-stimulated RAIU to the greatest extent among inhibitors examined in PCCl3 cells.

Small molecule inhibitors, such as Akti1/2 (Akt1/2 inhibitor), LY294002 (PI3K inhibitor), 17-AAG (Hsp90 inhibitor), PD98059 (MEK/ERK inhibitor), were examined for their effect on RAIU in PCCl3 cells under acute TSH-stimulated conditions. Cells withdrawn of TSH for 5 days have little RAIU activity and cells regain RAIU activity after addition of TSH for 24 hrs. As shown in **Figure 1**, all inhibitors examined significantly further increased TSH-stimulated RAIU activity. Among them, Akti1/2 increased TSH-stimulated RAIU to the greatest extent. Hence, we focused on Akti1/2 and searched for reagents that can further increase RAIU in Akti1/2 treated cells.

Among all inhibitors shown to increase RAIU, none of them can further increase RAIU in Akti1/2-treated PCCl3 cells. In FRTL-5 rat thyroid cells, TGF-β decreases RAIU (14, 15) and we have confirmed the same in PCCl3 cells (data not shown). TGF-β induces EMT (16-18), yet Akt inhibition decreases EMT (19-23). Taken together, Akti1/2 and TGF-β modulate both RAIU and EMT in an opposite manner. We thus hypothesized that EMT-associated genes may modulate RAIU in thyroid cells. We examined expression levels of 84 EMT-associated genes using EMT PCR array in cells treated with DMSO, Akti1/2 or TGF-β. The results were subjected to IPA analysis, and involucrin, a transglutaminase substrate protein, was predicted to be downregulated by Akti1/2 yet upregulated by TGF-β. Since involucrin expression appears to be inversely correlated with RAIU, we speculated that Apigenin, a plant-derived flavonoid known to inhibit involucrin expression in keratinocytes, may further increase Akti1/2-induced RAIU.

Apigenin further increases RAIU in Akti1/2-treated PCCl3 cells.

Apigenin (20 μM) alone had little effect on RAIU in PCCl3 cells. However, in combination with Akti1/2, Apigenin further increased RAIU in TSH-stimulated PCCl3 cells to a greater extent than Akti1/2 alone (**Figure 2A**). While Akti1/2 at 30 μM further increased RAIU compared to 10 μM (**Figure 2B**), cells had evident morphological changes. Apigenin at 50 μM did not significantly increase RAIU to a greater extent than 20 μM in the presence of either 10 μM or 30 μM Akti1/2. Accordingly, 10 μM Akti1/2 and 20 μM Apigenin were chosen for further studies. As shown in **Figure 2C**, 20 μM Apigenin decreased phospho-Akt level in PCCl3 cells as reported in many other cell types (24-28) and the combination of Apigenin and Akti1/2 further decreased phospho-Akt levels. The effect of Apigenin to further increase RAIU in cells treated with Akt inhibitor was confirmed in cells treated with 5 μM MK2206 (data not shown). MK2206 is another allosteric Akt inhibitor that is currently in clinical trials (29). Taken together, Apigenin alone had little effect on RAIU, yet it consistently further enhanced RAIU in PCCl3 cells in combination with Akt inhibitors.

Akt inhibition via shRNA knockdown or via hormonal modulation allows Apigenin to further increase RAIU in PCCl3 cells.

We further investigated whether Akt inhibition by other means would also allow Apigenin to increase RAIU in PCCl3 cells. RAIU was increased by ~1.7 fold in PCCl3 cells stably transfected with shAkt1/2 (**Figure 3A**), in which phospho-Akt level was evidently decreased (**Figure 3B**), compared to cells stably transfected with the control plasmid vector. Note that the relative molecular mass (M_r) of NIS protein was increased in cells treated with Apigenin in

combination with Akt inhibition. As expected, Apigenin further increased RAIU in PCCl3-shAkt1/2 cells, yet had little effect on RAIU in PCCl3-vector control cells.

It has been shown that phospho-Akt level is decreased in the absence of insulin in FRTL-5 rat thyroid cells under low-serum culture media (30). To recapitulate this culture condition, PCCl3 cells were deprived of TSH and insulin (4H) with 0.2% serum for 5 days, and then 4H culture media was replaced with 4H+TSH or 6H (4H+TSH+insulin) culture media for 24 hrs before adding either DMSO vehicle or Apigenin for an additional 24 hrs. As shown in **Figure 3C**, Apigenin did not increase RAIU in cells cultured in 4H or 6H condition, yet it did further increase RAIU in cells with 4H+TSH culture media. Note that phospho-Akt level in cells with 4H+TSH was lower than that in cells with 4H or 6H culture media (**Figure 3D**). Apigenin suppressed phospho-Akt levels in all three experimental groups and the extent of suppression is more pronounced in 0.2% serum than 5% serum conditions (**Figure 3D** vs. **Figure 2B**).

Since TSH is required for NIS expression, protein stability, and function, NIS protein is absent in cells under 4H conditions. Accordingly, Apigenin was not able to induce RAIU in the absence of TSH. The inability of Apigenin to increase RAIU under 6H conditions may be attributed to other effects of insulin rather than a higher phospho-Akt level prior to Apigenin addition. Taken together, Akt inhibition appears to be permissive for Apigenin to further increase RAIU, yet Apigenin's action to further enhance RAIU does not correlate with its effect on suppressing phospho-Akt levels.

Apigenin further increases iodide influx rate in Akti1/2-treated PCCl3 cells.

Since iodide uptake is mediated by NIS protein localized on the cell surface, we examined if Apigenin increases cell surface NIS protein levels in Akti1/2-treated PCCl3 cells. As shown in **Figure 4A**, Apigenin did not increase total or cell surface NIS protein levels in Akti1/2-treated cells. The absence of β-actin in cell surface fractions confirms the absence of cytosolic proteins. Because RAIU activity represents a steady state equilibrium between iodide influx and iodide efflux, we investigated whether Apigenin decreases iodide efflux rate in Akti1/2-treated cells. As shown in **Figure 4B**, Apigenin had little effect on the iodide efflux rate, which is different from the mechanism of 17-AAG (4).

Effects of Apigenin on maximum velocity of iodide influx (Vmax) and iodide affinity ($\propto 1/Km$) were evaluated. Similar to previous studies conducted on cells under chronic TSH stimulation (5), Akti1/2 alone increases Vmax by 1.44-fold compared to DMSO treated cells. In combination with Akti1/2, Apigenin further increased Vmax by 1.24-fold, compared to Akti1/2 treatment alone with little change in the Km value. Since Apigenin did not increase cell surface NIS protein levels, the increase in Vmax suggests that Apigenin increases the rate of iodide uptake by each NIS molecule on the cell surface.

Apigenin's effect to further increase RAIU in Akti1/2-treated cells requires p38 MAPK activity.

Since p38 MAPK is a well-known downstream effector of Apigenin (31-33), we examined if p38 MAPK mediates Apigenin's effect on RAIU in Akti1/2-treated PCCl3 cells. Pretreatment with an ATP-pocket binding inhibitor of p38MAPK, SB203580, for 1 hr and its continuous presence for 24 hrs diminished Apigenin's effect to further increase RAIU in Akti1/2-treated

cells. The fact that SB203580 treatment decreased RAIU in cells of all experimental groups indicates that p38MAPK activity plays a functional role to maintain optimal RAIU activity in PCCl3 cells. Apigenin's effect to further increase RAIU in Akti1/2-treated cells appears to be dependent, at least in part, upon p38 MAPK activity (**Figure 5A**).

SB203580 does not decrease the phosphorylation levels of p38 MAPK but inhibits its catalytic activity to phosphorylate its downstream substrates, such as MAPKAPK2. Using antibodies against MAPKAPK2, the non-phosphorylated form (lower band) can be resolved from the phosphorylated from (upper band). As shown in **Figure 5B**, MAPKAPK2 was predominantly in the phosphorylated form in acute TSH-stimulated PCCl3 cells, and the relative abundance of the non-phosphorylated form was increased by SB203580 treatment. The decrease of RAIU in SB203580-treated cells was not accompanied with a decrease in total NIS protein levels. Note that the relative molecular mass (M_r) of NIS protein was increased in cells treated with Apigenin in combination with Akt inhibition. Similar results were obtained using an allosteric p38 MAPK inhibitor, BIRB-796 (**Figure 5C** and **Figure 5D**). Using siRNA to knockdown either each individual or all four isoforms, p38MAPK was knocked down up to 60% and this was not sufficient to decrease p38 MAPK activity. Accordingly, neither increase in non-phosphorylated form of MAPKAPK2, nor decrease in RAIU, was observed (data not shown).

Apigenin's action to increase RAIU in Akti1/2-treated cells is independent of PKC-δ.

Another well-known downstream effector of Apigenin is PKC-δ (32, 33). To test if Apigenin modulates RAIU via PKC-δ, genetic knockdown of PKC-δ was performed using siRNAs. As shown in **Figure 6A**, neither RAIU in cells of all experimental groups, nor the

further increase of RAIU by Apigenin in Akti1/2-treated cells, was affected by PKC-δ knockdown. Western blot analysis confirmed that PKC-δ protein levels were considerably decreased by siRNA knockdown (**Figure 6B**). The total NIS protein level was modestly decreased by PKC-δ knockdown in acute TSH-stimulated PCCl3 cells, yet without accompanied decrease in RAIU activity.

Apigenin in combination with Akti1/2 further increases TSH-stimulated RAIU in BRAF^{V600E} expressing thyroid cells and thyroid tumor cells from TRβ$^{PV/PV}$ mice.

The BRAF^{V600E} oncogene is the most commonly found oncogene in papillary thyroid carcinomas that is associated with aggressive clinical features (34) and reduced radioiodine uptake (35). To ensure clinical relevance of this finding, we investigated whether Apigenin's effect on RAIU is reproducible in PCCl3 cells overexpressing the BRAF^{V600E} oncogene and in primary cultured cells derived from thyroid tumors of a preclinical mouse model.

As expected, BRAF^{V600E} induction via doxycycline resulted in decreased RAIU (**Figure 7A**) and decreased NIS protein levels (**Figure 7B**) in PCCl3/Tet-On BRAF^{V600E} cells. Akti1/2 alone increased RAIU in both un-induced and doxycycline-induced cells to much lesser extent (**Figure 7A**), compared to PCCl3 parental cells. Apigenin alone had little effect on RAIU; however, Apigenin was able to further increase RAIU in Akti1/2-treated cells (**Figure 7A**). Similar results were obtained in doxycycline-induced PCCl3/TetOn-PTC1 cells (data not shown). These results indicate that increased RAIU by combination treatment of Apigenin and Akti1/2 is not limited to non-transformed PCCl3 cells but also present in PCCl3 cells expressing oncogenes that are relevant to thyroid cancer patients.

TRβ$^{PV/PV}$ genetic-engineered mice develop thyroid tumors (36) that maintain NIS expression (37). It is of interest to note that combination treatment of Apigenin with Akti1/2 significantly increased RAIU in primary cultured cells derived from thyroid tumors developed in TRβ$^{PV/PV}$ mice (**Figure 7C**). Taken together, Apigenin in combination with Akti1/2 increases RAIU in established rat thyroid cell lines as well as primary cultured cells of thyroid tumors from a preclinical thyroid cancer mouse model.

Discussion

In PCCl3 rat thyroid cells, we show that acute TSH-stimulated RAIU is enhanced by various inhibitors targeting signaling nodes over-activated in thyroid tumors. Among them, Akti1/2 increased RAIU to the greatest extent, and Apigenin further increased RAIU in Akti1/2-treated thyroid cells. This action of Apigenin was not mediated by an increase in cell surface NIS protein levels or a decrease in iodide efflux rate. Instead, Apigenin increased iodide influx rate in Akti1/2-treated cells, and this effect required p38MAPK activity. Most importantly, the increase in TSH-stimulated RAIU activity by Apigenin in combination with Akti1/2 was confirmed in BRAF^{V600E} expressing PCCl3 cells as well as in primary cultured tumor cells from TRβ$^{PV/PV}$ genetic-engineered mice.

Akti1/2 alone increased iodide influx rate, and Apigenin in combination with Akti1/2 further increased iodide influx rate (**Figure 4C**). To this date, molecular mechanisms underlying the modulation of iodide influx rate mediated by NIS remain unknown. Phosphorylation has been shown to modulate the activity of many transporters, such as glucose transporter (38) and Na^+/H^+

Exchanger isoform-1 (39). NIS is a phosphorylated protein (40) and several phosphorylation sites have been identified (41). An increase of M_r in NIS protein was noted in cells treated with Apigenin in combination with either Akt inhibitor or Akt knockdown in parental and $BRAF^{V600E}$ expressing PCCl3 cells. In these experimental settings, the increase of M_r in NIS was accompanied with an increase in RAIU. It will be of interest to determine whether the increase of M_r reflects NIS phosphorylation and whether NIS phosphorylation leads to increased iodide influx rate in thyroid cells.

However, an increase of M_r in NIS did not always translate into increased RAIU. As shown in **Figure 5**, one hour pre-treatment of p38 MAPK inhibitor followed by its continuous presence for 24 hrs diminished Apigenin's effect to further increase RAIU in Akti1/2-treated cells despite that the increase of M_r in NIS remains unchanged. Conversely, iodide influx rate can be upregulated without an increase of M_r in NIS, as in the case of cells treated with Akti1/2 alone. Taken together, NIS-mediated iodide influx rate may be modulated by multiple mechanisms.

Akt inhibition alone, via inhibitor or shRNA knockdown, further increased TSH-stimulated RAIU in PCCl3 cells. Apigenin alone also decreased phospho-Akt levels, yet with little effect on thyroidal RAIU activity. This discrepancy may be explained by a lesser decrease in phospho-Akt levels by Apigenin than Akti1/2 or shAkt1/2. Accordingly, one may speculate that a threshold of Akt inhibition needs to be achieved to increase RAIU, and that a further increase in RAIU by Apigenin in Akti1/2-treated cells was mediated by further Akt inhibition. However, Akt inhibition is not always accompanied with increased RAIU. For example, the evident decrease in phospho-Akt levels by Apigenin treatment was not accompanied with increased RAIU in PCCl3

cells with 0.2% serum culture media. Furthermore, SB203580 diminished Apigenin's effect to further increase RAIU in Akti1/2 treated cells without evident change in phospho-Akt levels. Finally, it is of interest to note that an increase of M_r in NIS only occurred in cells treated with Apigenin+Akt inhibition. This suggests that Apigenin's action to further increase RAIU in Akti1/2 treated cells may not be fully explained by its effect on further Akt inhibition.

SB203580 decreased TSH-induced *NIS* mRNA levels, when SB203580 and TSH were added at the same time to TSH-deprived FRTL-5 cells (42, 43). In this study, a p38 MAPK inhibitor was added 24 hrs after acute TSH stimulation, when NIS mRNA and protein were already synthesized. Neither SB203580 nor BIRB-796 altered steady state NIS protein levels, yet both resulted in drastic decrease in RAIU. Accordingly, p38 MAPK activity is not only required for TSH-stimulated NIS expression but also required for optimal NIS-mediated RAIU activity. However, the action of Akti1/2 on RAIU is independent of p38 MAPK activity, as neither SB203580 nor BIRB-796 affected the fold of increase in RAIU by Akti1/2 alone.

Matowe et al. reported that PKC-δ disappeared within 24 hrs of TSH withdrawal and reappeared 24 hrs after TSH addition in FRTL-5 rat thyroid cells (44). Our study shows that steady state NIS protein levels were modestly decreased upon PKC-δ knockdown, yet without accompanied change in RAIU. This discrepancy may be explained by an absent change in cell surface NIS protein levels or by an increased NIS activity that compensates the decreased NIS protein levels. Neither Akti1/2-induced RAIU nor Apigenin's ability to further increase Akti1/2-induced RAIU was dependent upon PKC-δ.

The increase in RAIU by Apigenin+Akti1/2 was reproduced in $BRAF^{V600E}$ or RET/PTC1 expressing thyroid cells. Apigenin+Akti1/2 also increased RAIU in thyroid tumor cells from $TR\beta^{PV/PV}$ mice. Accordingly, Apigenin+Akti1/2 may increase TSH-stimulated RAIU in various thyroid tumors that carry the $BRAF^{V600E}$ mutation, RET/PTC1 rearrangement, or PI3K overactivation. A recent study reported a synergistic effect between Apigenin and an Akt inhibitor on cytotoxic effects of PLX4032-treated anaplastic thyroid carcinoma cells (45). It would also be of interest to further examine whether other flavonoids with a similar structure as Apigenin would have the same effect.

In summary, our studies suggest that Apigenin as a dietary supplement that, along with Akt inhibitors, can further enhance the efficacy of radioiodine therapy for thyroid cancer patients. Several Akt inhibitors, such as MK-2206 or GSK2110183, are currently in oncological pipelines to treat other types of cancer. Our finding has potential for translation into clinical trials upon confirmation in preclinical thyroid cancer mouse models.

Acknowledgements: This work was supported by NIH grant PO1-CA-124570. We appreciate MP Brandt for dissecting thyroid tumors, and Dr. M Ostrowski, Dr. B Kaur and A Suresh for scientific inputs.

Author Disclosure Statement: No competing financial interests exist.

Address all correspondence to: Dr. Sissy M. Jhiang, 1645 Neil Ave, 304 Hamilton Hall, Columbus, OH 43210. Tel.: (614) 292-4312; Fax: (614) 292-4888; E-mail: jhiang.1@osu.edu

References:

1. Pacini F, Ladenson PW, Schlumberger M, Driedger A, Luster M, Kloos RT, Sherman S, Haugen B, Corone C, Molinaro E, Elisei R, Ceccarelli C, Pinchera A, Wahl RL, Leboulleux S, Ricard M, Yoo J, Busaidy NL, Delpassand E, Hanscheid H, Felbinger R, Lassmann M, Reiners C 2006 Radioiodine ablation of thyroid remnants after preparation with recombinant human thyrotropin in differentiated thyroid carcinoma: results of an international, randomized, controlled study. J Clin Endocrinol Metab **91**:926-932.

2. Molinaro E, Giani C, Agate L, Biagini A, Pieruzzi L, Bianchi F, Brozzi F, Ceccarelli C, Viola D, Piaggi P, Vitti P, Pacini F, Elisei R 2013 Patients With Differentiated Thyroid Cancer Who Underwent Radioiodine Thyroid Remnant Ablation With Low-Activity 131I After Either Recombinant Human TSH or Thyroid Hormone Therapy Withdrawal Showed the Same Outcome After a 10-Year Follow-up. J Clin Endocrinol Metab **98**:2693-2700.

3. Vadysirisack DD, Venkateswaran A, Zhang Z, Jhiang SM 2007 MEK signaling modulates sodium iodide symporter at multiple levels and in a paradoxical manner. Endocr Relat Cancer **14**:421-432.

4. Marsee DK, Venkateswaran A, Tao H, Vadysirisack D, Zhang Z, Vandre DD, Jhiang SM 2004 Inhibition of heat shock protein 90, a novel RET/PTC1-associated protein, increases radioiodide accumulation in thyroid cells. J Biol Chem **279**:43990-43997.

5. Liu YY, Zhang X, Ringel MD, Jhiang SM 2012 Modulation of sodium iodide symporter expression and function by LY294002, Akti-1/2 and Rapamycin in thyroid cells. Endocr Relat Cancer **19**:291-304.

6. Liu D, Hu S, Hou P, Jiang D, Condouris S, Xing M 2007 Suppression of BRAF/MEK/MAP kinase pathway restores expression of iodide-metabolizing genes in thyroid cells expressing the V600E BRAF mutant. Clin Cancer Res **13**:1341-1349.

7. Chakravarty D, Santos E, Ryder M, Knauf JA, Liao XH, West BL, Bollag G, Kolesnick R, Thin TH, Rosen N, Zanzonico P, Larson SM, Refetoff S, Ghossein R, Fagin JA 2011 Small-molecule MAPK inhibitors restore radioiodine incorporation in mouse thyroid cancers with conditional BRAF activation. J Clin Invest **121**:4700-4711.

8. Liu YY, Brandt MP, Shen DH, Kloos RT, Zhang X, Jhiang SM 2011 Single photon emission computed tomography imaging for temporal dynamics of thyroidal and salivary radionuclide accumulation in 17-allyamino-17-demothoxygeldanamycin-treated thyroid cancer mouse model. Endocr Relat Cancer **18**:27-37.

9. Ho AL, Grewal RK, Leboeuf R, Sherman EJ, Pfister DG, Deandreis D, Pentlow KS, Zanzonico PB, Haque S, Gavane S, Ghossein RA, Ricarte-Filho JC, Dominguez JM, Shen R, Tuttle RM, Larson SM, Fagin JA 2013 Selumetinib-enhanced radioiodine uptake in advanced thyroid cancer. N Engl J Med **368**:623-632.

10. Mitsutake N, Knauf JA, Mitsutake S, Mesa C, Jr., Zhang L, Fagin JA 2005 Conditional BRAFV600E expression induces DNA synthesis, apoptosis, dedifferentiation, and chromosomal instability in thyroid PCCL3 cells. Cancer Res **65**:2465-2473.

11. Kaneshige M, Kaneshige K, Zhu X, Dace A, Garrett L, Carter TA, Kazlauskaite R, Pankratz DG, Wynshaw-Boris A, Refetoff S, Weintraub B, Willingham MC, Barlow C, Cheng S 2000 Mice with a targeted mutation in the thyroid hormone beta receptor gene exhibit impaired growth and resistance to thyroid hormone. Proc Natl Acad Sci U S A **97**:13209-13214.

12. Gordi T, Khamis H 2004 Simple solution to a common statistical problem: interpreting multiple tests. Clin Ther **26**:780-786.

13. Holm S 1979 A simple sequentially rejective multiple test procedure. Scand J Stat **6** 65–70.

14. Costamagna E, Garcia B, Santisteban P 2004 The functional interaction between the paired domain transcription factor Pax8 and Smad3 is involved in transforming growth factor-beta repression of the sodium/iodide symporter gene. J Biol Chem **279**:3439-3446.

15. Nicolussi A, D'Inzeo S, Santulli M, Colletta G, Coppa A 2003 TGF-beta control of rat thyroid follicular cells differentiation. Mol Cell Endocrinol **207**:1-11.

16. Riesco-Eizaguirre G, Rodriguez I, De la Vieja A, Costamagna E, Carrasco N, Nistal M, Santisteban P 2009 The BRAFV600E oncogene induces transforming growth factor beta secretion leading to sodium iodide symporter repression and increased malignancy in thyroid cancer. Cancer Res **69**:8317-8325.

17. Vasko V, Espinosa AV, Scouten W, He H, Auer H, Liyanarachchi S, Larin A, Savchenko V, Francis GL, de la Chapelle A, Saji M, Ringel MD 2007 Gene expression and functional evidence of epithelial-to-mesenchymal transition in papillary thyroid carcinoma invasion. Proc Natl Acad Sci U S A **104**:2803-2808.

18. Knauf JA, Sartor MA, Medvedovic M, Lundsmith E, Ryder M, Salzano M, Nikiforov YE, Giordano TJ, Ghossein RA, Fagin JA Progression of BRAF-induced thyroid cancer is associated with epithelial-mesenchymal transition requiring concomitant MAP kinase and TGFbeta signaling. Oncogene **30**:3153-3162.

19. Chen W, Wu S, Zhang G, Wang W, Shi Y 2013 Effect of AKT inhibition on epithelial-mesenchymal transition and ZEB1-potentiated radiotherapy in nasopharyngeal carcinoma. Oncology letters **6**:1234-1240.

20. Chen WC, Lai YA, Lin YC, Ma JW, Huang LF, Yang NS, Ho CT, Kuo SC, Way TD 2013 Curcumin Suppresses Doxorubicin-Induced Epithelial-Mesenchymal Transition via the Inhibition of TGF-beta and PI3K/AKT Signaling Pathways in Triple-Negative Breast Cancer Cells. J Agric Food Chem. Advance online publication. doi: 10.1021/jf404092f.

21. Chen WF, Gao WD, Li QL, Zhou PH, Xu MD, Yao LQ 2013 SLIT2 inhibits cell migration in colorectal cancer through the AKT-GSK3beta signaling pathway. Int J Colorectal Dis **28**:933-940.

22. Hong KO, Kim JH, Hong JS, Yoon HJ, Lee JI, Hong SP, Hong SD 2009 Inhibition of Akt activity induces the mesenchymal-to-epithelial reverting transition with restoring E-cadherin expression in KB and KOSCC-25B oral squamous cell carcinoma cells. J Exp Clin Cancer Res **28**:28.

23. Qian X, Anzovino A, Kim S, Suyama K, Yao J, Hulit J, Agiostratidou G, Chandiramani N, McDaid HM, Nagi C, Cohen HW, Phillips GR, Norton L, Hazan RB 2013 N-cadherin/FGFR promotes metastasis through epithelial-to-mesenchymal transition and stem/progenitor cell-like properties. Oncogene. Advance online publication. doi: 10.1038/onc.2013.310.

24. Kaur P, Shukla S, Gupta S 2008 Plant flavonoid apigenin inactivates Akt to trigger apoptosis in human prostate cancer: an in vitro and in vivo study. Carcinogenesis **29**:2210-2217.

25. Van Dross RT, Hong X, Pelling JC 2005 Inhibition of TPA-induced cyclooxygenase-2 (COX-2) expression by apigenin through downregulation of Akt signal transduction in human keratinocytes. Mol Carcinog **44**:83-91.

26. Yuan WX, XiuJuan Zhao, XiaoHua Zhang, Ling Zhang, Kun Wu 2007 Inhibition of PKB/Akt activity involved in apigenin-induced apoptosis in human gastric carcinoma cells. Chinese Sci Bull **52**:2226-2232.

27. Way TD, Kao MC, Lin JK 2004 Apigenin induces apoptosis through proteasomal degradation of HER2/neu in HER2/neu-overexpressing breast cancer cells via the phosphatidylinositol 3-kinase/Akt-dependent pathway. J Biol Chem **279**:4479-4489.

28. Fang J, Xia C, Cao Z, Zheng JZ, Reed E, Jiang BH 2005 Apigenin inhibits VEGF and HIF-1 expression via PI3K/AKT/p70S6K1 and HDM2/p53 pathways. FASEB J **19**:342-353.

29. Yap TA, Yan L, Patnaik A, Fearen I, Olmos D, Papadopoulos K, Baird RD, Delgado L, Taylor A, Lupinacci L, Riisnaes R, Pope LL, Heaton SP, Thomas G, Garrett MD, Sullivan DM, de Bono JS, Tolcher AW 2011 First-in-man clinical trial of the oral pan-AKT inhibitor MK-2206 in patients with advanced solid tumors. J Clin Oncol **29**:4688-4695.

30. Saito J, Kohn AD, Roth RA, Noguchi Y, Tatsumo I, Hirai A, Suzuki K, Kohn LD, Saji M, Ringel MD 2001 Regulation of FRTL-5 thyroid cell growth by phosphatidylinositol (OH) 3 kinase-dependent Akt-mediated signaling. Thyroid **11**:339-351.

31. Kim SH, Kang JG, Kim CS, Ihm SH, Choi MG, Yoo HJ, Lee SJ 2013 Apigenin induces c-Myc-mediated apoptosis in FRO anaplastic thyroid carcinoma cells. Mol Cell Endocrinol **369**:130-139.

32. Gonzalez-Mejia ME, Voss OH, Murnan EJ, Doseff AI 2010 Apigenin-induced apoptosis of leukemia cells is mediated by a bimodal and differentially regulated residue-specific phosphorylation of heat-shock protein-27. Cell Death Dis **1**:e64.

33. Vargo MA, Voss OH, Poustka F, Cardounel AJ, Grotewold E, Doseff AI 2006 Apigenin-induced-apoptosis is mediated by the activation of PKCdelta and caspases in leukemia cells. Biochem Pharmacol **72**:681-692.

34. Kim SJ, Lee KE, Myong JP, Park JH, Jeon YK, Min HS, Park SY, Jung KC, Koo do H, Youn YK 2012 BRAF V600E mutation is associated with tumor aggressiveness in papillary thyroid cancer. World J Surg **36**:310-317.

35. Mian C, Barollo S, Pennelli G, Pavan N, Rugge M, Pelizzo MR, Mazzarotto R, Casara D, Nacamulli D, Mantero F, Opocher G, Busnardo B, Girelli ME 2008 Molecular characteristics in papillary thyroid cancers (PTCs) with no 131I uptake. Clin Endocrinol (Oxf) **68**:108-116.

36. Suzuki H, Willingham MC, Cheng SY 2002 Mice with a mutation in the thyroid hormone receptor beta gene spontaneously develop thyroid carcinoma: a mouse model of thyroid carcinogenesis. Thyroid **12**:963-969.

37. Ying H, Suzuki H, Zhao L, Willingham MC, Meltzer P, Cheng SY 2003 Mutant thyroid hormone receptor beta represses the expression and transcriptional activity of peroxisome proliferator-activated receptor gamma during thyroid carcinogenesis. Cancer Res **63**:5274-5280.

38. Bourque S, Lemoine R, Sequeira-Legrand A, Fayolle L, Delrot S, Pugin A 2002 The elicitor cryptogein blocks glucose transport in tobacco cells. Plant Physiol **130**:2177-2187.

39. Rigor RR, Damoc C, Phinney BS, Cala PM 2011 Phosphorylation and activation of the plasma membrane Na+/H+ exchanger (NHE1) during osmotic cell shrinkage. PLoS One **6**:e29210.

40. Riedel C, Levy O, Carrasco N 2001 Post-transcriptional regulation of the sodium/iodide symporter by thyrotropin. J Biol Chem **276**:21458-21463.

41. Vadysirisack DD, Chen ES, Zhang Z, Tsai MD, Chang GD, Jhiang SM 2007 Identification of in vivo phosphorylation sites and their functional significance in the sodium iodide symporter. J Biol Chem **282**:36820-36828.

42. Pomerance M, Abdullah HB, Kamerji S, Correze C, Blondeau JP 2000 Thyroid-stimulating hormone and cyclic AMP activate p38 mitogen-activated protein kinase cascade. Involvement of protein kinase A, rac1, and reactive oxygen species. J Biol Chem **275**:40539-40546.

43. Kogai T, Ohashi E, Jacobs MS, Sajid-Crockett S, Fisher ML, Kanamoto Y, Brent GA 2008 Retinoic acid stimulation of the sodium/iodide symporter in MCF-7 breast cancer cells is mediated by the insulin growth factor-I/phosphatidylinositol 3-kinase and p38 mitogen-activated protein kinase signaling pathways. J Clin Endocrinol Metab **93**:1884-1892.

44. Matowe WC, Gupta S, Ginsberg J 1996 Regulation of protein kinase C isoforms in FRTL-5 thyroid cells by TSH and phorbol ester. Thyroid **6**:53-58.

45. Kim SH, Kang JG, Kim CS, Ihm SH, Choi MG, Yoo HJ, Lee SJ 2013 Akt inhibition enhances the cytotoxic effect of apigenin in combination with PLX4032 in anaplastic thyroid carcinoma cells harboring BRAFV600E. J Endocrinol Invest. Advance online publication. doi: 10.3275/9099.

Figure legends:

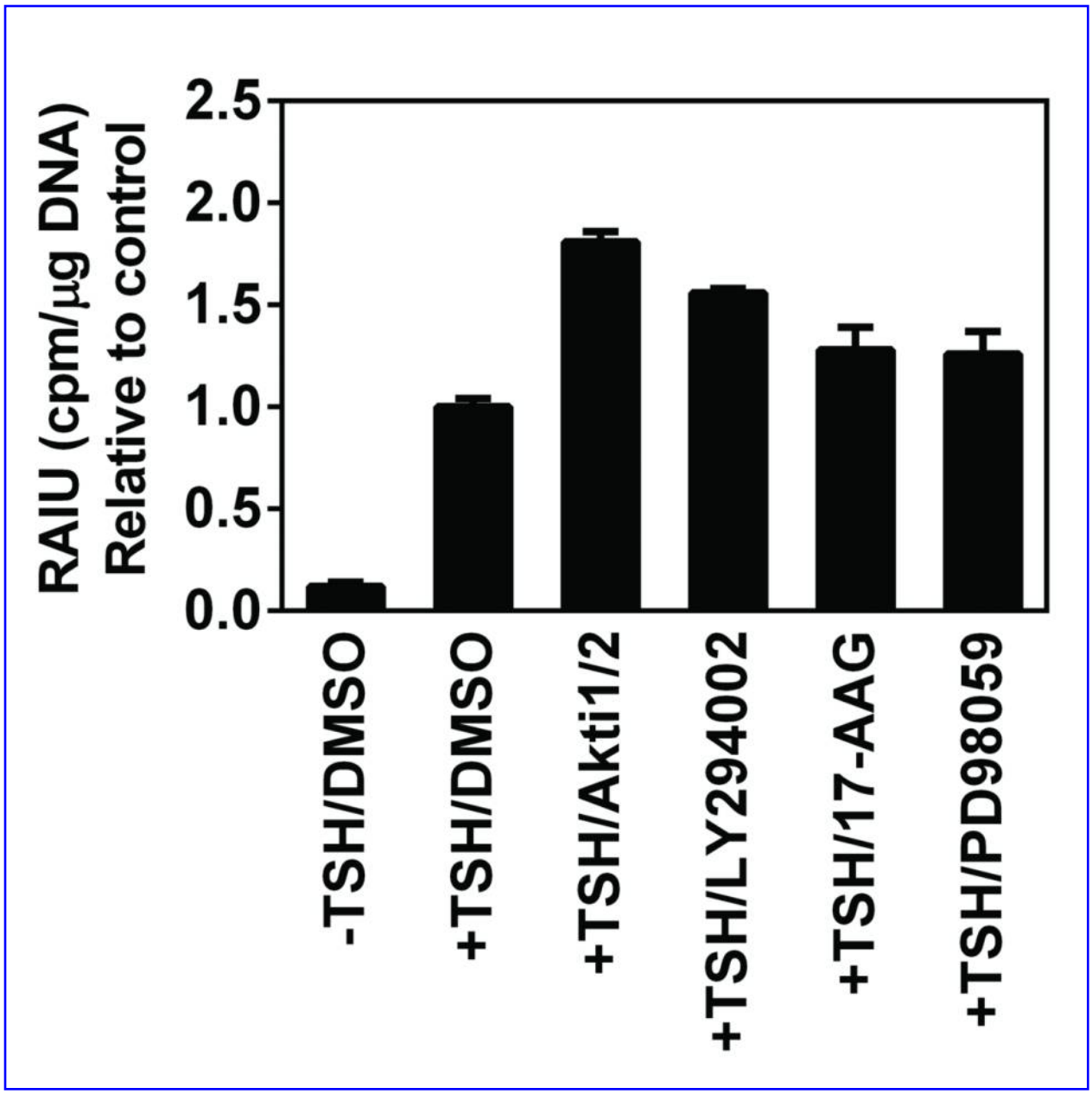


Figure 1. TSH-stimulated RAIU in PCCl3 cells can be further increased by various reagents. Cells were withdrawn from TSH (-TSH) for 5 days and then stimulated with TSH (+TSH) for 24 hrs followed by treatment with 10 µl DMSO (vehicle control), 10 µM Akti1/2, 10 µM LY294002, 3 µM 17-AAG or 40 µM PD98059 for 24 hrs before RAIU analysis. RAIU in +TSH/DMSO treated cells was significantly higher than in –TSH/DMSO-treated cells. RAIU in

TSH-stimulated cells treated with Akti1/2, LY294002, 17-AAG and PD98059 was significantly higher than in DMSO-treated cells. Data are expressed as mean + SD (n=3).

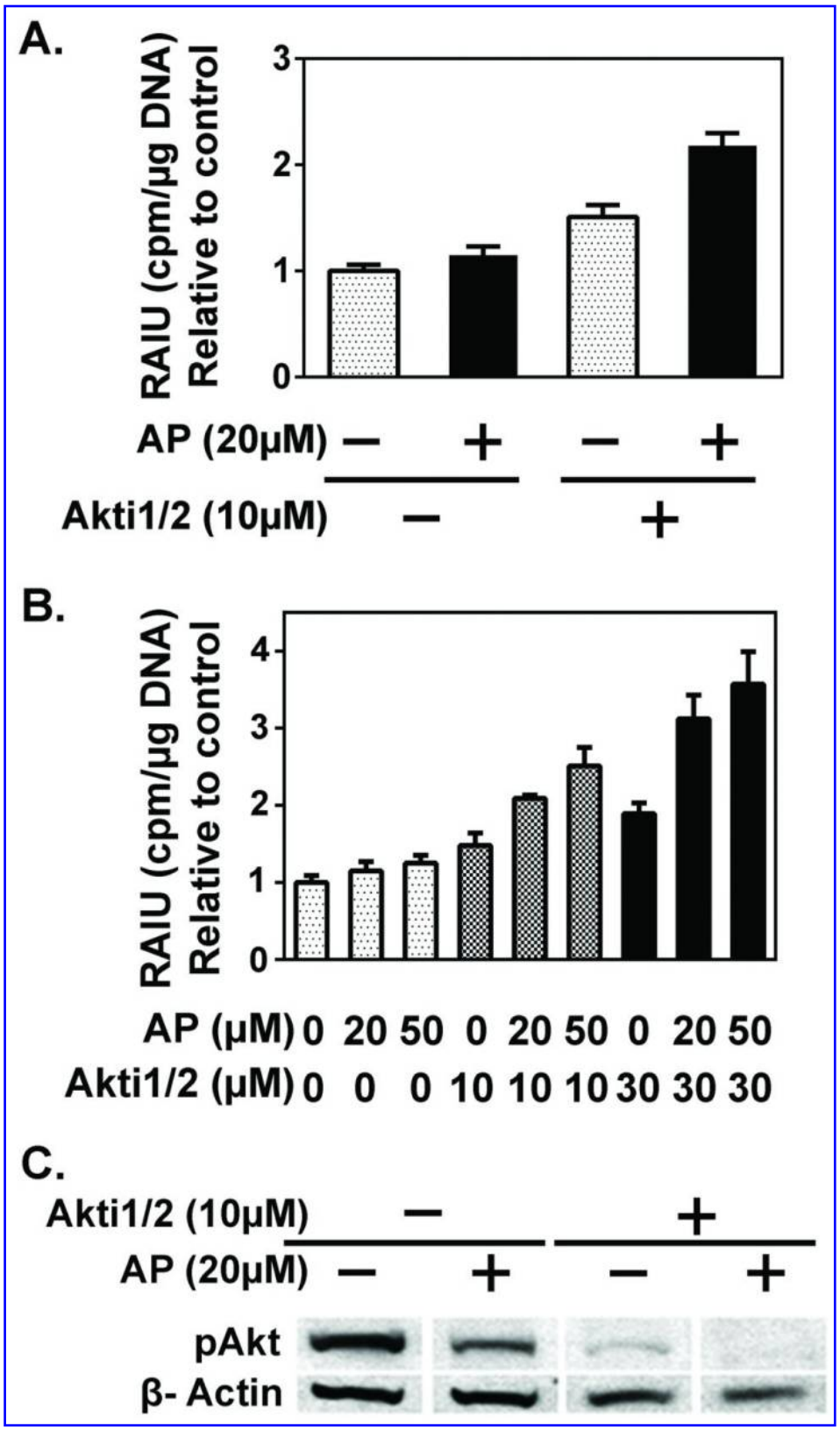


Figure 2. Apigenin further increases RAIU activity in Akti1/2-treated PCCl3 rat thyroid cells. (**A**) RAIU in Apigenin (AP)-treated cells was not significantly higher than in DMSO-treated cells, yet RAIU in AP+Akti1/2-treated cells was significantly higher than in Akti1/2-treated cells. RAIU in Akti1/2-treated cells was significantly higher than in DMSO-treated cells. (**B**) RAIU in cells treated with a combination of 20 μM AP and 10 μM or 30 μM Akti1/2 is significantly higher than RAIU in cells treated with 10 μM or 30 μM Akti1/2 alone. At 50 μM, AP did not significantly further increase RAIU compared to 20 μM AP in the presence or absence of Akti1/2 treatment. RAIU in cells treated with Akti1/2 at 10 μM and 30 μM were significantly higher than in DMSO-treated control. (**C**) Western blots show that phospho-Akt levels are decreased in cells treated with AP, Akti1/2, or AP+Akti1/2. Total Akt levels remained the same (data not shown). β-actin served as a loading control. For panels (A) and (B), data are expressed as mean + SD (n=3). All cells were acutely stimulated with TSH for 24 hr prior to treatments.

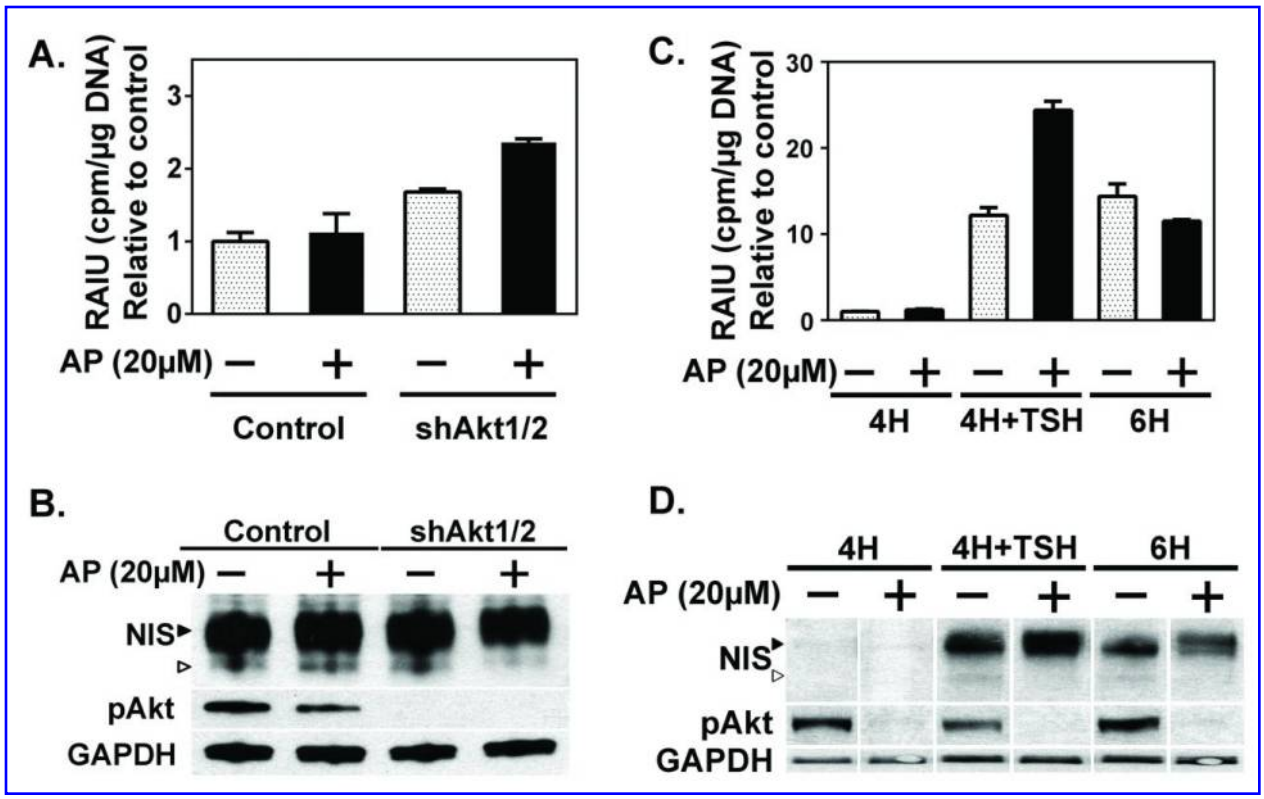
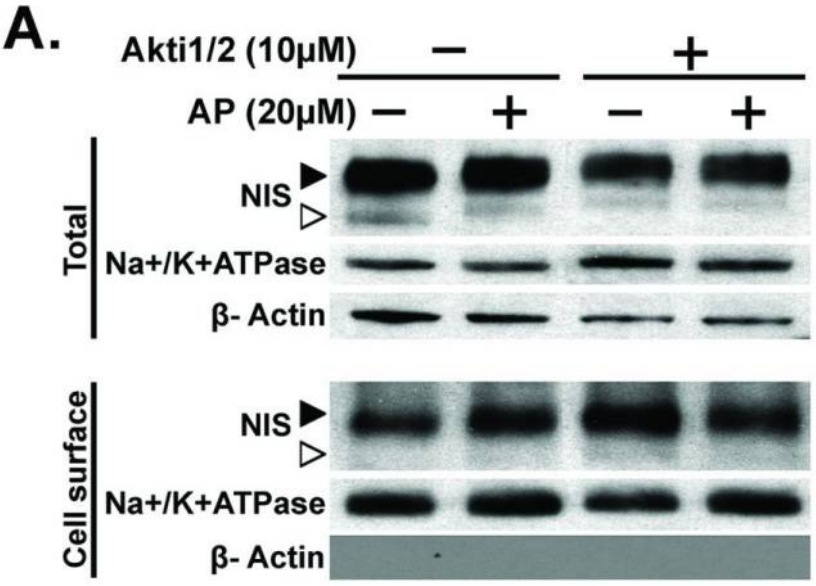
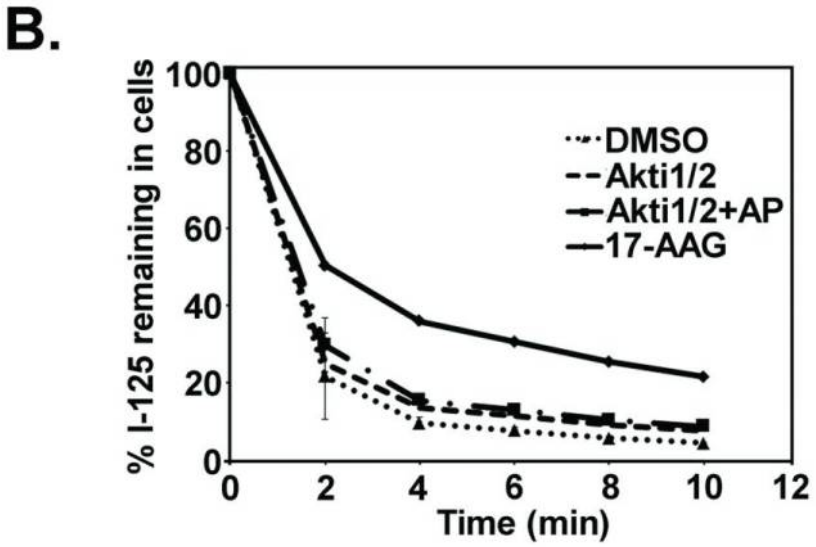


Figure 3. Akt inhibition appears to be permissive for Apigenin to enhance RAIU in PCCl3 cells. (**A**) RAIU is significantly increased by AP treatment in shAkt1/2 stably-transfected cells but not in vector-transfected cells. RAIU in shAkt1/2-stably transfected cells is significantly higher than RAIU in vector-transfected cells. (**B**) Western blots show that phospho-Akt levels are decreased in shAkt1/2 stably-transfected cells due to effective knockdown of total Akt (data not shown). Note an increase in M_r of NIS in shAkt1/2 transfected cells treated with AP. (**C**) RAIU in AP-treated cells is greater than in DMSO-treated cells under 4H+TSH conditions but not under 4H or 6H conditions. Cells were deprived of TSH and insulin (4H) with 0.2% serum for 5 days, and then 4H culture media was replaced with 4H+TSH or 6H (4H+TSH+insulin) culture media for 24 hrs before adding either DMSO vehicle or AP for an additional 24 hrs. (**D**) Western blots show that phospho-Akt level in cells with 4H+TSH is lower than that in cells

treated with 4H or 6H media, and AP suppressed phospho-Akt levels. NIS is undetectable in cells under 4H media but was evidently increased in cells under 4H+TSH and 6H. Total Akt levels remained the same in all lanes (data not shown). For panels (A) and (C), data are expressed as mean + SD (n=3). In panels (B) and (D), GAPDH served as a loading control. Hyperglycosylated NIS is indicated with (▶) and hypoglycosylated NIS with (▷).





C.

Treatment	Vmax (pmol/min/μg DNA)	Km (μM)
DMSO	5.10±0.99	37.80±14.00
Akti1/2	7.35±0.21	47.45±3.04
Apigenin+Akti1/2	9.10±0.14	40.15±1.63

Figure 4. Apigenin increases iodide influx rate in Akti1/2-treated PCCl3 cells. (**A**) Western blots show that total or cell surface NIS protein levels are not changed by AP in Akti1/2-treated cells. Exposure times for total and cell surface NIS were 2 and 10 minutes respectively. Hyperglycosylated NIS is indicated with (►) and hypoglycosylated NIS with (▷). Na^+/K^+ ATPase served as a loading control for cell surface proteins and β-actin for total cell lysates. Enrichment of cell surface proteins was confirmed by the absence of β-actin. (**B**) Efflux assays show that neither Akti1/2 nor AP+Akti1/2 treatment significantly decrease the iodide efflux rate compared to DMSO treatment. 17-AAG, known to significantly decrease the iodide efflux rate, was used as the positive control. The iodide efflux rate is shown as the percentage of iodide remaining in the cells plotted at 2 min intervals. (**C**) Iodide kinetics assay show that the maximum velocity of iodide influx (Vmax) was increased by AP+Akti1/2 compared to Akti1/2 alone, yet there is little change in the Km value. Vmax and Km from two independent experiments are expressed as average + range. All cells were acutely stimulated with TSH for 24 hr prior to treatments.

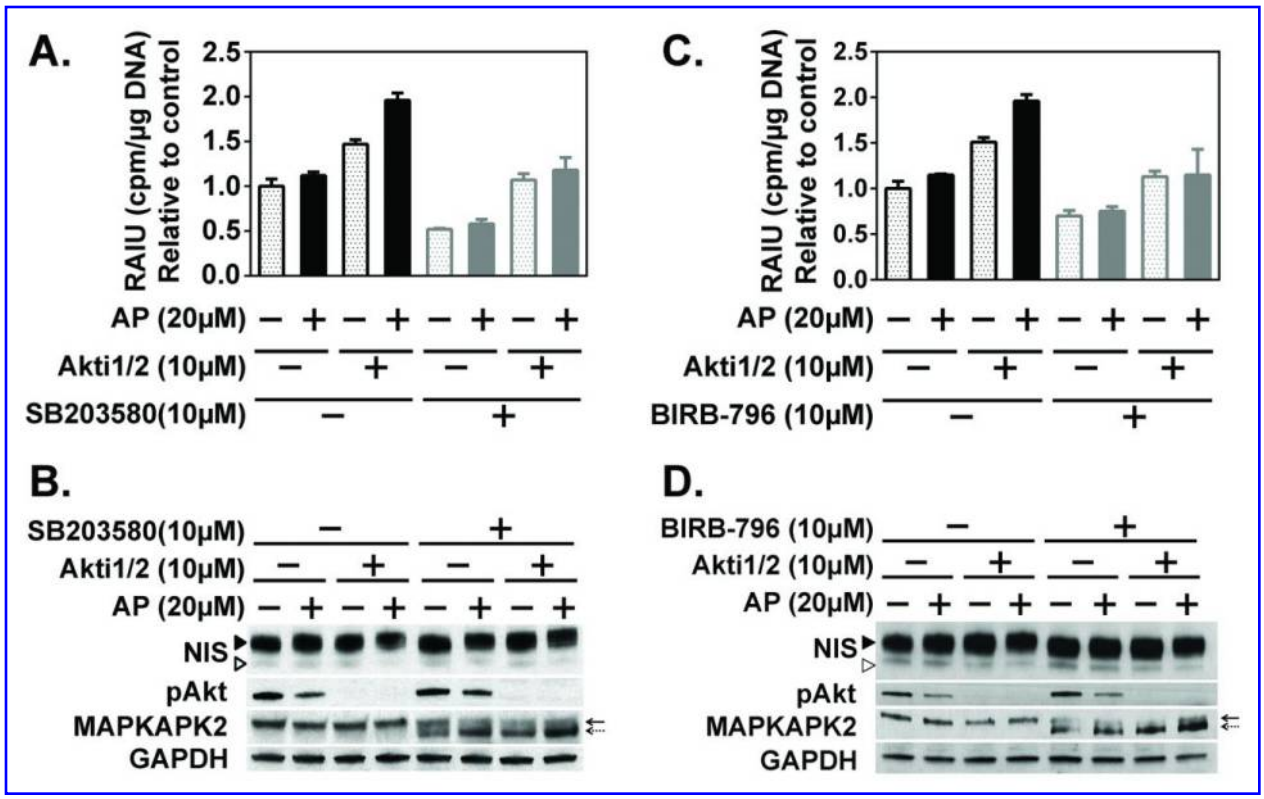


Figure 5. Apigenin's effect to further increase RAIU in Akti1/2-treated PCCl3 cells requires p38 MAPK activity. (**A**) In the presence of SB203580, RAIU in cells treated with DMSO, AP, Akti1/2 and AP+Akti1/2 is significantly decreased and AP does not significantly increase RAIU in Akti1/2-treated cells. (**B**) Western blots show an increase in the relative abundance of non-phosphorylated MAPKAPK2, an immediate downstream effector of p38 MAPK, thereby confirming the inhibition of p38 MAPK by SB203580. The NIS protein level is not decreased by SB203580. Note an increase in M_r of NIS in cells treated with AP+Akti1/2 in the presence or absence of SB203580. (**C, D**) Similar results were obtained using an allosteric p38 MAPK inhibitor, BIRB-796. In panels (A) and (C), data are expressed as mean + SD (n=3). In panels (B) and (D), GAPDH served as a loading control. Arrows ← and <-- indicate

phosphorylated and non-phosphorylated MAPKAPK2, respectively. Hyperglycosylated NIS is indicated with (▶) and hypoglycosylated NIS with (▷). All cells were acutely stimulated with TSH for 24 hr prior to treatments.

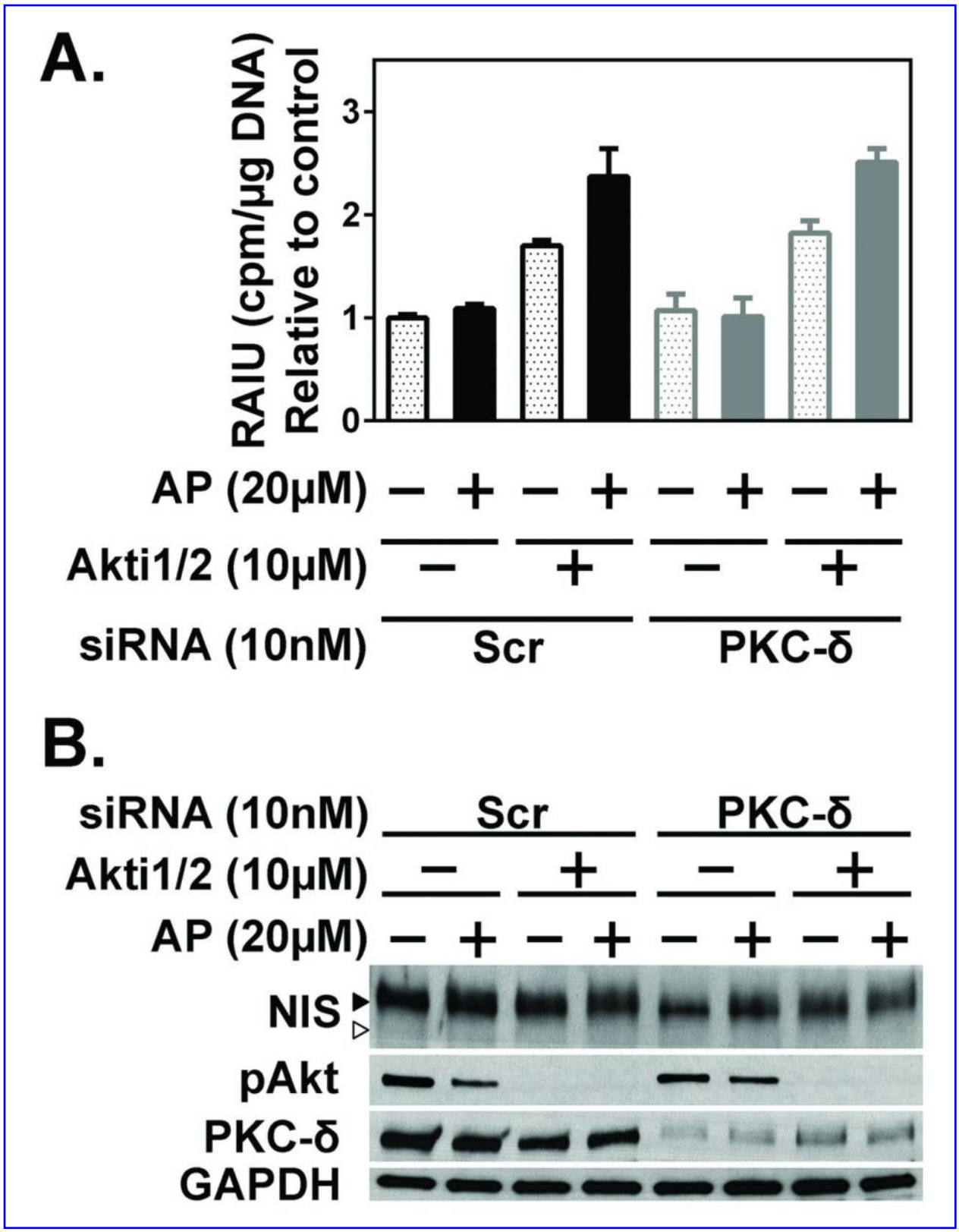


Figure 6. Apigenin's effect to further increase RAIU in Akti1/2-treated PCCl3 cells is independent of PKC-δ. Cells were transfected with 10 nM of scrambled (Scr) or PKC-δ siRNA for 24 hrs followed by treatment with reagents for additional 24 hrs. (**A**) RAIU in Scr siRNA-transfected cells was not significantly different from PKC-δ siRNA-transfected cells. Data are expressed as mean + SD (n=3). (**B**) PKC-δ knockdown was confirmed by evident decrease in PKC-δ protein level in Western blots. NIS protein level was moderately decreased by PKC-δ knockdown. Hyperglycosylated NIS is indicated with (▶) and hypoglycosylated NIS with (▷). GAPDH served as a loading control.

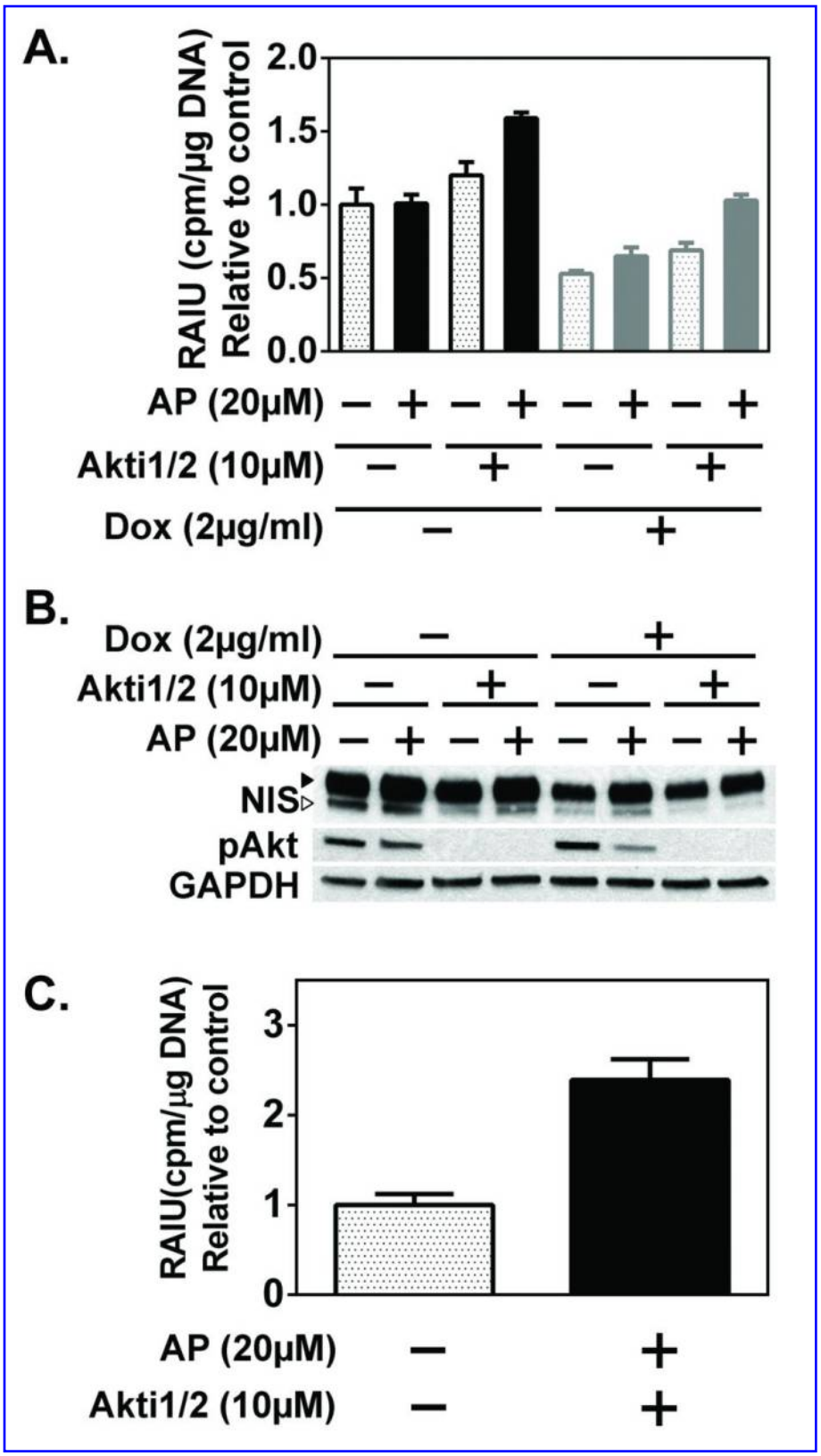


Figure 7. Apigenin in combination with Akti1/2 increases RAIU in BRAF expressing PCCl3 cells and in thyroid tumor cells from TRβ$^{PV/PV}$ mice. (**A**) RAIU in doxycycline-induced cells is significantly decreased compared to uninduced cells. RAIU in AP+Akti1/2-treated cells is significantly higher than Akti1/2-treated cells in both uninduced and induced cells. (**B**) Western blots show that total NIS protein levels are decreased nearly by half in BRAF^{V600E} expressing cells. Hyperglycosylated NIS is indicated with (▶) and hypoglycosylated NIS with (▷). Note an increase in M_r of NIS by AP+Akti1/2 in both uninduced and induced cells. GAPDH served as a loading control. (**C**) Primary cultured thyroid tumor cells from TRβ$^{PV/PV}$ mice were treated with DMSO or AP+Akti1/2, one day post-seeding. RAIU in AP+Akti1/2-treated cells was significantly greater than RAIU in DMSO treated cells. For (A) and (C), data are expressed as mean + SD (n=3).